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Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
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R C van Dijk

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CropDesign N.V.
Technologiepark
9052 Zwijnaarde 3
BELGIQUE

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Plants having modified growth characteristics and method for making the same

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Plants having modified growth characteristics and method for making the same

Field of the invention

The present invention concerns a method for modifying plant growth characteristics. More specifically, the present invention concerns a method for modifying plant growth characteristics by modulating expression of a nucleic acid sequence encoding a metallothionein and/or activity of a metallothionein in a plant. The present invention also concerns plants having modulated expression of a nucleic acid sequence encoding a metallothionein and/or modulated activity of a metallothionein, which plants have modified growth characteristics relative to corresponding wild type plants.

Background of the invention

Some heavy metals, particularly copper and zinc, are essential micronutrients that play a role in a range of plant physiological processes via the action of Cu- and Zn-dependent enzymes. These and other nonessential heavy metal ions, such as cadmium, lead, and mercury, are highly reactive and consequently can be toxic to living cells. Thus plants, like all living organisms, have evolved a suite of mechanisms that control and respond to the uptake and accumulation of both essential and nonessential heavy metals. These mechanisms include the chelation and sequestration of heavy metals by particular ligands. The two best-characterized heavy metal-binding ligands in plant cells are the phytochelatins (PCs) and metallothioneins (MTs). MTs are cysteine-rich polypeptides encoded by a family of genes. In contrast, PCs are a family of enzymatically synthesized cysteine-rich peptides.

Metallothioneins, products of mRNA translation, are low molecular weight, cysteine-rich, metal-binding proteins. MT proteins and genes are found throughout the animal and plant kingdoms as well as in the prokaryote *Synechococcus*. The large number of cysteine residues in MTs bind a variety of metals by mercaptide bonds. MTs typically contain two metal-binding, cysteine-rich domains that give these metalloproteins a dumbbell conformation. The data available today tends to support a role for MTs in copper tolerance (as PCs protect against cadmium).

The classification of MT proteins is based on the arrangement of Cys residues. Cobbett and Goldsbrough (Annu Rev Plant Biol, 53 159-82, 2002) discriminate four classes: Type 1 to Type 4. Type 2 MTs contain two cysteine rich domains separated by a spacer of approximately 40 amino acids, with the first pair of cysteines present as a Cys-Cys motif in amino acid positions

3 and 4. In addition, the sequences of the N-terminal domain of Type 2 MTs (MSCCGGNCGCS-) are highly conserved.

Arabidopsis, rice and sugarcane contain genes encoding all four types of MTs. General observations can be made about the expression pattern of these genes. Type 4 MTs are restricted to developing seeds. Type 1 MT expression tends to be higher in roots than shoots, whereas generally the reverse is observed for Type 2 MTs. Type 3 MTs are expressed in fleshy fruits or in leaves of non-fleshy fruit producing plants (like *Arabidopsis*). Various environmental factors influence the expression of these genes.

Transgenics plants expressing GUS under the control of the AtMT2a promoter have been produced. In young plants, staining was found only in cotyledons and lateral root tips. GUS activity in older plants was seen at the base of trichomes, in hydathodes, sepals, anthers, stigma, root tips, and vascular tissues at branch points of lateral roots. As leaves aged, GUS staining increased.

Transgenic plants expressing MTs have been produced serving two purposes:

1) Phytoremediation of heavy metal contaminated soils: transgenic tobacco and *Brassica* plants, engineered to overexpress MTs, moderately resist the toxicity of heavy metals like cadmium (Suh *et al.*, Mol Cells, 8 (6) 678 – 684, 1998).

2) Nutritional quality improvement, when co-expressed in rice with phytase and ferritin, to improve iron diet in humans. The metallothionein-like protein, rich in cysteine, (a sulfur-rich amino acid) helps in iron absorption by the human digestive system (Potrykus I (2002) Nutritional improvement of rice to reduce malnutrition in developing countries. In "Plant Biotechnology 2002 and Beyond" I.K. Vasil (ed.) pp 401-406).

Given the ever-increasing world population, it remains a major goal of agricultural research to improve the efficiency of agriculture and to increase the diversity of plants in horticulture. Conventional means for crop and horticultural improvements utilise selective breeding techniques to identify plants having desirable characteristics. However, such selective breeding techniques have several drawbacks, namely that these techniques are typically labour intensive and result in plants that often contain heterogeneous genetic complements that may not always result in the desirable trait being passed on from parent plants. Advances in molecular biology have allowed mankind to manipulate the germplasm of animals and plants. Genetic engineering of plants entails the isolation and manipulation of genetic material (typically in the form of DNA or RNA) and the subsequent introduction of that genetic material into a plant. Such technology has led to the development of plants having various improved economic, agronomic or horticultural traits. A trait of particular economic interest is high yield.

Detailed description

Therefore according to a first embodiment of the present invention there is provided a method for modifying the growth characteristics of a plant, comprising modulating expression in a plant of a nucleic acid sequence encoding a metallothionein and/or modulating activity in a plant of a metallothionein.

Modulating (enhancing or decreasing) expression of a nucleic acid sequence encoding a metallothionein or modulation of the activity of the metallothionein itself encompasses altered expression of a gene and/or altered levels of a gene product, namely a polypeptide, in specific cells or tissues.

Advantageously, modulation of expression of a nucleic acid sequence encoding a metallothionein and/or modulation of activity of the metallothionein itself may be effected by chemical means, i.e. by exogenous application of one or more compounds or elements capable of modulating activity of the metallothionein and/or capable of modulating expression of a metallothionein gene (which may be either an endogenous gene or a transgene introduced into a plant). The exogenous application may comprise contacting or administering cells, tissues, organs or organisms with the gene product or a homologue, derivative or active fragment thereof and/or to antibodies to the gene product. Such antibodies may comprise "plantibodies", single chain antibodies, IgG antibodies and heavy chain camel antibodies, as well as fragments thereof. Modulation of expression of a nucleic acid sequence encoding a metallothionein and/or modulation of activity of the metallothionein itself may also be effected as a result of decreased levels of factors that directly or indirectly activate or inactivate a metallothionein. Additionally or alternatively, contacting or administering cells, tissues, organs or organisms with an interacting protein or to an inhibitor or activator of the gene product provides another exogenous means for modulation of expression of a nucleic acid sequence encoding a metallothionein and/or for modulation of activity of the metallothionein itself.

Therefore, according to one aspect of the present invention, there is provided a method for modifying the growth characteristics of a plant, comprising exogenous application of one or more compounds or elements capable of modulating expression of a metallothionein gene and/or capable of modulating activity of a metallothionein.

Additionally or alternatively, and according to a preferred embodiment of the present invention, modulation of expression of a nucleic acid sequence encoding a metallothionein and/or modulation of activity of the metallothionein itself may be effected by recombinant means.

Such recombinant means may comprise a direct and/or indirect approach for modulation of expression of a nucleic acid sequence and/or for modulation of the activity of a protein.

For example, an indirect approach may comprise introducing, into a plant, a nucleic acid sequence capable of modulating activity of the protein in question (a metallothionein) and/or expression of the gene in question (a gene encoding a metallothionein). The metallothionein gene or the metallothionein protein may be wild type, i.e. the native or endogenous nucleic acid or polypeptide. Alternatively, it may be a nucleic acid derived from the same or another species, which gene is introduced as a transgene, for example by transformation. This transgene may be substantially modified from its native form in composition and/or genomic environment through deliberate human manipulation. Also encompassed by an indirect approach for modulating activity of a metallothionein and/or expression of a metallothionein gene is the inhibition or stimulation of regulatory sequences that drive expression of the native gene or transgene. Such regulatory sequences may be introduced into a plant.

A direct and preferred approach on the other hand comprises introducing into a plant a nucleic acid sequence encoding a metallothionein or a homologue, derivative or active fragment thereof. The nucleic acid sequence may be introduced into a plant by, for example, transformation. The nucleic acid sequence may be derived (either directly or indirectly (if subsequently modified)) from any source provided that the sequence, when expressed in a plant, leads to modulated expression of a metallothionein nucleic acid/gene or modulated activity of a metallothionein. The nucleic acid sequence may be isolated from a microbial source, such as bacteria, yeast or fungi, or from a plant, algal or animal (including human) source. This nucleic acid may be substantially modified from its native form in composition and/or genomic environment through deliberate human manipulation. The nucleic acid sequence is preferably a homologous nucleic acid sequence, i.e. a nucleic acid sequence obtained from a plant, whether from the same plant species or different. The nucleic acid sequence is isolated from a dicotyledonous species, preferably from the family Brassicaceae, further preferably from *Arabidopsis thaliana*. More preferably, the nucleic acid is as represented by SEQ ID NO: 1 or a portion thereof or a nucleic acid sequence capable of hybridising therewith or is a nucleic acid sequence encoding an amino acid sequence represented by SEQ ID NO: 2 or a homologue, derivative or active fragment thereof.

The term metallothionein nucleic acid sequence/gene, as defined herein, refers to a nucleic acid sequence as represented by SEQ ID NO: 1 or a portion thereof or to nucleic acid sequences capable of hybridising therewith, which hybridising sequences encode proteins having metallothionein activity, i.e. similar biological activity to that of SEQ ID NO: 1, and to

nucleic acid sequences encoding an amino acid sequence represented by SEQ ID NO: 2 or a homologue, derivative or active fragment thereof. The protein encoded by SEQ ID NO 2 belongs to the group of Type 2 metallothioneins (Cobbett and Goldsbrough, Annu Rev Plant Biol, 53 159-82, 2002).

Advantageously, the method according to the present invention may also be practised using portions of a sequence represented by SEQ ID NO: 1 or by using sequences that hybridise (preferably under stringent conditions) to SEQ ID NO: 1 (which hybridising sequences encode proteins having AtMT2a activity), or by using homologues, derivatives or active fragments of a sequence according to SEQ ID NO: 2. Suitable homologues of SEQ ID NO 2 include proteins from *Arabidopsis thaliana* as represented in accession nos SMMUL or AAM62956 or protein from *Brassica* sp. as represented in accession nos Q42494 or AAF70558.

Methods for the search and identification of metallothionein homologues would be well within the realm of a person skilled in the art. Methods for the alignment of sequences for comparison are well known in the art, such methods include GAP, BESTFIT, BLAST, FASTA and TFASTA. GAP uses the algorithm of Needleman and Wunsch (J. Mol. Biol. 48: 443-453, 1970) to find the alignment of two complete sequences that maximises the number of matches and minimises the number of gaps. The BLAST algorithm calculates percent sequence identity and performs a statistical analysis of the similarity between the two sequences. The software for performing BLAST analysis is publicly available through the National Centre for Biotechnology Information. The above-mentioned homologues were identified using BLAST default parameters.

"Homologues" of a metallothionein protein encompass peptides, oligopeptides, polypeptides, proteins and enzymes having amino acid substitutions, deletions and/or insertions relative to the unmodified protein in question and having similar biological and functional activity as the unmodified protein from which they are derived. To produce such homologues, amino acids of the protein may be replaced by other amino acids having similar properties (such as similar hydrophobicity, hydrophilicity, antigenicity, propensity to form or break α-helical structures or β-sheet structures). Conservative substitution tables are well known in the art (see for example Creighton (1984) Proteins. W.H. Freeman and Company). The homologues useful in the method according to the invention have at least 50% sequence identity or similarity (functional identity) to the unmodified protein, alternatively at least 60% sequence identity or similarity to an unmodified protein, alternatively at least 70% sequence identity or similarity to an unmodified protein. Typically, the homologues have at least 80% sequence identity or similarity to an unmodified protein, preferably at least 85% sequence identity or similarity, further

preferably at least 90% sequence identity or similarity to an unmodified protein, most preferably at least 95% sequence identity or similarity to an unmodified protein. Preferred homologues include the proteins represented by accession numbers X62818 (SEQ ID NO 3) and SMMUL (SEQ ID NO 4).

Two special forms of homology, orthologous and paralogous, are evolutionary concepts used to describe ancestral relationships of genes. The term "paralogous" relates to gene-duplications within the genome of a species leading to paralogous genes. The term "orthologous" relates to homologous genes in different organisms due to ancestral relationship. The term "homologues" as used herein also encompasses paralogues and orthologues of the proteins useful in the methods according to the invention.

"Substitutional variants" of a protein are those in which at least one residue in an amino acid sequence has been removed and a different residue inserted in its place. Amino acid substitutions are typically of single residues, but may be clustered depending upon functional constraints placed upon the polypeptide; insertions will usually be of the order of about 1-10 amino acid residues, and deletions will range from about 1-20 residues. Preferably, amino acid substitutions comprise conservative amino acid substitutions.

"Insertional variants" of a protein are those in which one or more amino acid residues are introduced into a predetermined site in a protein. Insertions can comprise amino-terminal and/or carboxy-terminal fusions as well as intra-sequence insertions of single or multiple amino acids. Generally, insertions within the amino acid sequence will be smaller than amino- or carboxy-terminal fusions, of the order of about 1 to 10 residues. Examples of amino- or carboxy-terminal fusion proteins or peptides include the binding domain or activation domain of a transcriptional activator as used in the yeast two-hybrid system, phage coat proteins, $(histidine)_6$-tag, glutathione S-transferase-tag, protein A, maltose-binding protein, dihydrofolate reductase, Tag·100 epitope, c-myc epitope, FLAG®-epitope, lacZ, CMP (calmodulin-binding peptide), HA epitope, protein C epitope and VSV epitope.

"Deletion variants" of a protein are characterised by the removal of one or more amino acids from the protein. Amino acid variants of a protein may readily be made using peptide synthetic techniques well known in the art, such as solid phase peptide synthesis and the like, or by recombinant DNA manipulations. Methods for the manipulation of DNA sequences to produce substitution, insertion or deletion variants of a protein are well known in the art. For example, techniques for making substitution mutations at predetermined sites in DNA are well known to those skilled in the art and include M13 mutagenesis, T7-Gen in vitro mutagenesis (USB,

Cleveland, OH), QuickChange Site Directed mutagenesis (Stratagene, San Diego, CA), PCR-mediated site-directed mutagenesis or other site-directed mutagenesis protocols.

The term "derivatives" refers to peptides, oligopeptides, polypeptides, proteins and enzymes which may comprise substitutions, deletions or additions of naturally and non-naturally occurring amino acid residues compared to the amino acid sequence of a naturally-occurring form of the protein, for example, as presented in SEQ ID NO: 2. "Derivatives" of a metallothionein encompass peptides, oligopeptides, polypeptides, proteins and enzymes which may comprise naturally occurring altered, glycosylated, acylated or non-naturally occurring amino acid residues compared to the amino acid sequence of a naturally-occurring form of the polypeptide. A derivative may also comprise one or more non-amino acid substituents compared to the amino acid sequence from which it is derived, for example a reporter molecule or other ligand, covalently or non-covalently bound to the amino acid sequence such as, for example, a reporter molecule which is bound to facilitate its detection, and non-naturally occurring amino acid residues relative to the amino acid sequence of a naturally-occurring protein.

"Active fragments" of a metallothionein encompasses at least five contiguous amino acid residues of a protein, which residues retain similar biological and/or functional activity to the naturally occurring protein.

Advantageously, the method according to the present invention may also be practised using portions of a DNA or nucleic acid sequence, which portions retain metallothionein activity, i.e. a similar biological function to that of SEQ ID NO: 2. Portions of a DNA sequence refer to a piece of DNA derived or prepared from an original (larger) DNA molecule, which DNA portion, when expressed in a plant, gives rise to plants having modified growth characteristics. The portion may comprise many genes, with or without additional control elements, or may contain just spacer sequences etc.

The present invention also encompasses nucleic acid sequences capable of hybridising with a nucleic acid sequence encoding a metallothionein, which nucleic acid sequences may also be useful in practising the methods according to the invention. The term "hybridisation" as defined herein is a process wherein substantially homologous complementary nucleotide sequences anneal to each other. The hybridisation process can occur entirely in solution, i.e. both complementary nucleic acids are in solution. Tools in molecular biology relying on such a process include the polymerase chain reaction (PCR; and all methods based thereon), subtractive hybridisation, random primer extension, nuclease S1 mapping, primer extension,

reverse transcription, cDNA synthesis, differential display of RNAs, and DNA sequence determination. The hybridisation process can also occur with one of the complementary nucleic acids immobilised to a matrix such as magnetic beads, Sepharose beads or any other resin. Tools in molecular biology relying on such a process include the isolation of poly (A+) mRNA. The hybridisation process can furthermore occur with one of the complementary nucleic acids immobilised to a solid support such as a nitro-cellulose or nylon membrane or immobilised by e.g. photolithography to, for example, a siliceous glass support (the latter known as nucleic acid arrays or microarrays or as nucleic acid chips). Tools in molecular biology relying on such a process include RNA and DNA gel blot analysis, colony hybridisation, plaque hybridisation, in situ hybridisation and microarray hybridisation. In order to allow hybridisation to occur, the nucleic acid molecules are generally thermally or chemically denatured to melt a double strand into two single strands and/or to remove hairpins or other secondary structures from single stranded nucleic acids. The stringency of hybridisation is influenced by conditions such as temperature, salt concentration and hybridisation buffer composition. High stringency conditions for hybridisation include high temperature and/or low salt concentration (salts include NaCl and Na_3-citrate) and/or the inclusion of formamide in the hybridisation buffer and/or lowering the concentration of compounds such as SDS (sodium dodecyl sulphate) in the hybridisation buffer and/or exclusion of compounds such as dextran sulphate or polyethylene glycol (promoting molecular crowding) from the hybridisation buffer. Conventional hybridisation conditions are described in, for example, Sambrook (2001) Molecular Cloning: a laboratory manual, 3rd Edition Cold Spring Harbor Laboratory Press, CSH, New York, but the skilled man will appreciate that numerous different hybridisation conditions can be designed in function of the known or the expected homology and/or length of the nucleic acid sequence. Specifically hybridising refers to hybridising under stringent conditions, i.e. at a temperature of 60°C followed by washes in 2XSSC, 0.1XSDS, and 1X SSC, 0.1X SDS. Sufficiently low stringency hybridisation conditions are particularly preferred for the isolation of nucleic acids homologous to the DNA sequences of the invention defined supra. Elements contributing to homology include allelism, degeneration of the genetic code and differences in preferred codon usage.

The methods according to the present invention may also be practised using an alternative splice variant of a nucleic acid sequence encoding a metallothionein protein. The term "alternative splice variant" as used herein encompasses variants of a nucleic acid sequence in which selected introns and/or exons have been excised, replaced or added. Such variants will be ones in which the biological activity of the protein remains unaffected, which can be achieved by selectively retaining functional segments of the protein. Such splice variants may be found in nature or can be manmade. Methods for making such splice variants are well

known in the art. Therefore according to another aspect of the present invention, there is provided, a method for modifying the growth characteristics of plants, comprising modulating expression in a plant of an alternative splice variant of a nucleic acid sequence encoding a metallothionein protein and/or by modulating activity of a metallothionein protein encoded by the alternative splice variant. Preferably, the splice variant is a splice variant of the sequence represented by SEQ ID NO: 1 or SEQ ID NO: 3.

Advantageously, the methods according to the present invention may also be practised using allelic variants of a nucleic acid sequence encoding a metallothionein protein, preferably an allelic variant of a sequence represented by SEQ ID NO: 1 or SEQ ID NO: 3. Allelic variants exist in nature and encompassed within the methods of the present invention is the use of these natural alleles. The use of these allelic variants in particular conventional breeding programmes, such as in marker-assisted breeding is also encompassed by the present invention; this may be in addition to their use in the methods according to the present invention. Such breeding programmes sometimes require the introduction of allelic variations in the plants by mutagenic treatment of a plant. One suitable mutagenic method is EMS mutagenesis. Identification of allelic variants then takes place by, for example, PCR. This is followed by a selection step for selection of superior allelic variants of the sequence in question and which give rise to altered growth characteristics in a plant. Selection is typically carried out by monitoring growth performance of plants containing different allelic variants of the sequence in question, for example, different allelic variants of SEQ ID NO: 1. Monitoring growth performance can be done in a greenhouse or in the field. Further optional steps include crossing plants, in which the superior allelic variant was identified, with another plant. This could be used, for example, to make a combination of interesting phenotypic features. Allelic variants also encompass Single Nucleotide Polymorphisms (SNPs), as well as Small Insertion/Deletion Polymorphisms (INDELs). The size of INDELs is usually less than 100 bp. SNPs and INDELs form the largest set of sequence variants in naturally occurring polymorphic strains of most organisms.

According to another aspect of the present invention, advantage may be taken of the nucleotide sequence capable of modulating expression of a nucleic acid encoding a metallothionein in breeding programmes. The nucleic acid sequence may be on a chromosome, or a part thereof, comprising at least the nucleic acid sequence encoding the metallothionein and preferably also one or more related family members. In an example of such a breeding programme, a DNA marker is identified which may be genetically linked to a gene capable of modulating expression of a nucleic acid encoding a metallothionein in a plant, which gene may be a gene encoding the metallothionein itself or any other gene which may

directly or indirectly influence expression of the gene encoding a metallothionein and/or activity of the metallothionein itself. This DNA marker may then used in breeding programs to select plants having altered growth characteristics.

The methods according to the present invention may also be practised by introducing into a plant at least a part of a (natural or artificial) chromosome (such as a Bacterial Artificial Chromosome (BAC)), which chromosome contains at least a gene/nucleic acid sequence encoding a metallothionein (such as SEQ ID NO: 1 or SEQ ID NO: 3), preferably together with one or more related gene family members. Therefore, according to a further aspect of the present invention, there is provided a method for modifying the growth characteristics of plants by introducing into a plant at least a part of a chromosome comprising at least a gene/nucleic acid encoding a metallothionein.

According to one aspect of the present invention, enhanced or increased expression of a nucleic acid is envisaged. Methods for obtaining enhanced or increased expression of genes or gene products are well documented in the art and include, for example, overexpression driven by a strong promoter, the use of transcription enhancers or translation enhancers. Preferably, the nucleic acid to be overexpressed encodes a metallothionein, further preferably the nucleic acid sequence encoding the metallothionein is isolated from a dicotyledonous plant, preferably of the family Brassicaceae, further preferably wherein the sequence is isolated from *Arabidopsis thaliana*, most preferably the nucleic acid sequence is as represented by SEQ ID NO: 1 or a portion thereof, or encodes an amino acid sequence as represented by SEQ ID NO: 2 or a homologue, derivative or active fragment thereof. Alternatively, the nucleic acid sequence encoding the metallothionein is as represented by SEQ ID NO: 3 or is a portion thereof, or encodes an amino acid sequence as represented by SEQ ID NO: 4 or encodes a homologue, derivative or active fragment thereof. It should be noted that the applicability of the invention is not limited to use of the nucleic acid represented by SEQ ID NO: 1 nor to the nucleic acid sequence encoding the amino acid sequence of SEQ ID NO: 2, but that other nucleic acid sequences encoding homologues, derivatives or active fragments of SEQ ID NO: 2, or portions of SEQ ID NO: 1, or sequences hybridising with SEQ ID NO: 1 may be used in the methods of the present invention.

According to another aspect of the present invention, decreased expression of a nucleic acid sequence is envisaged. Modulating gene expression (whether by a direct or indirect approach) encompasses altered transcript levels of a gene. Altered transcript levels can be sufficient to induce certain phenotypic effects, for example via the mechanism of cosuppression. Here the overall effect of overexpression of a transgene is that there is less activity in the cell of the

protein encoded by a native gene having homology to the introduced transgene. Other examples of decreasing expression are also well documented in the art and include, for example, downregulation of expression by anti-sense techniques, co-suppression techniques, RNAi techniques, small interference RNAs (siRNAs), microRNA (miRNA), the use of ribozymes, etc. Therefore according to a particular aspect of the invention, there is provided a method for modulating growth characteristics of plants, including technologies that are based on the synthesis of antisense transcripts, complementary to the mRNA of a metallothionein gene, or based on RNA interference. Advantageously, the methods according to the present invention may also be practised by downregulation of a nucleic acid sequence encoding a metallothionein protein. Plants having modified growth characteristics may be obtained by expressing a nucleic acid sequence encoding a metallothionein in either sense or antisense orientation. Techniques for downregulation are well known in the art. The terms "gene silencing" or "downregulation" of expression, as used herein, refer to lowering levels of gene expression and/or levels of active gene product and/or levels of gene product activity. Such decreases in expression may be accomplished by, for example, the addition of coding sequences or parts thereof in a sense orientation (if it is desired to achieve co-suppression). Therefore, according to one aspect of the present invention, the growth of a plant may be modified by introducing into a plant an additional copy (in full or in part) of a metallothionein gene already present in a host plant. The additional gene will silence the endogenous gene, giving rise to a phenomenon known as co-suppression.

Genetic constructs aimed at silencing gene expression may comprise the metallothionein nucleotide sequence, for example as represented by SEQ ID NO: 1 (or one or more portions thereof) in a sense and/or antisense orientation relative to the promoter sequence. The sense or antisense copies of at least part of the endogenous gene in the form of direct or inverted repeats may be utilised in the methods according to the invention. The growth characteristics of plants may also be modified by introducing into a plant at least part of an antisense version of the nucleotide sequence represented, for example, by SEQ ID NO: 1. It should be clear that part of the nucleic acid (a portion) could achieve the desired result. Homologous anti-sense genes are preferred to heterologous anti-sense genes, homologous genes being plant genes, preferably plant genes from the same plant species, and heterologous genes being genes from non-plant species.

Another method for downregulation of gene expression or gene silencing comprises use of ribozymes, for example as described in Atkins *et al.* 1994 (WO 94/00012), Lenee *et al.* 1995 (WO 95/03404), Lutziger *et al.* 2000 (WO 00/00619), Prinsen *et al.* 1997 (WO 97/3865) and Scott *et al.* 1997 (WO 97/38116).

089-MT-PROV

Gene silencing may also be achieved by insertion mutagenesis (for example, T-DNA insertion or transposon insertion) or by gene silencing strategies as described by, among others, Angell and Baulcombe 1998 (WO 98/36083), Lowe *et al.* 1989 (WO 98/53083), Lederer *et al.* 1999 (WO 99/15682) or Wang *et al.* 1999 (WO 99/53050). Expression of an endogenous gene may also be reduced if the endogenous gene contains a mutation. Such a mutant gene may be isolated and introduced into the same or different plant species in order to obtain plants having modified growth characteristics.

According to a second embodiment of the present invention, genetic constructs and vectors to facilitate introduction and/or expression of the nucleotide sequences useful in the methods according to the invention are provided. Therefore, according to a second embodiment of the present invention, there is provided a gene construct comprising:

(i) a nucleic acid sequence capable of modulating expression of a nucleic acid encoding a metallothionein and/or activity of a metallothionein protein;

(ii) one or more control sequences capable of driving expression of the nucleic acid sequence of (i); and optionally

(iii) a transcription termination sequence.

Constructs useful in the methods according to the present invention may be constructed using recombinant DNA technology well known to persons skilled in the art. The gene constructs may be inserted into vectors, which may be commercially available, suitable for transforming into plants and suitable for expression of the gene of interest in the transformed cells.

The nucleic acid sequence capable of modulating expression of a nucleic acid encoding a metallothionein and/or activity of the metallothionein itself may be a nucleic acid sequence encoding a metallothionein or a homologue, derivative or active fragment thereof, such as any of the nucleic acid sequences described hereinbefore. A preferred nucleic acid sequence is the sequence represented by SEQ ID NO: 1 or a portion thereof or sequences capable of hybridising therewith or a nucleic acid sequence encoding a sequence represented by SEQ ID NO: 2 or a homologue, derivative or active fragment thereof.

Plants are transformed with a vector comprising the sequence of interest (i.e., the nucleic acid sequence capable of modulating expression of a nucleic acid encoding a metallothionein), which sequence is operably linked to one or more control sequences (at least a promoter). The terms "regulatory element", "control sequence" and "promoter" are all used herein interchangeably and are to be taken in a broad context to refer to regulatory nucleic acid

sequences capable of effecting expression of the sequences to which they are ligated. Encompassed by the aforementioned terms are transcriptional regulatory sequences derived from a classical eukaryotic genomic gene (including the TATA box which is required for accurate transcription initiation, with or without a CCAAT box sequence) and additional regulatory elements (i.e. upstream activating sequences, enhancers and silencers) which alter gene expression in response to developmental and/or external stimuli, or in a tissue-specific manner. Also included within the term is a transcriptional regulatory sequence of a classical prokaryotic gene, in which case it may include a –35 box sequence and/or –10 box transcriptional regulatory sequences. The term "regulatory element" also encompasses a synthetic fusion molecule or derivative which confers, activates or enhances expression of a nucleic acid molecule in a cell, tissue or organ. The terms "control sequence", "regulatory sequence", "regulatory element" and "promoter" are used interchangeably herein. The term "operably linked" as used herein refers to a functional linkage between the promoter sequence and the gene of interest, such that the promoter sequence is able to initiate transcription of the gene of interest.

Advantageously, any type of promoter may be used to drive expression of the nucleic acid sequence depending on the desired outcome. For example, a meristem-specific promoter, such as the rnr (ribonucleotide reductase), cdc2a promoter and the cyc07 promoter, could be used to effect expression in all growing parts of the plant, thereby increasing cell proliferation, which in turn would increase yield or biomass. If the desired outcome would be to influence seed characteristics, such as the storage capacity, seed size, seed number, biomass etc., then a seed-specific promoter, such as p2S2, pPROLAMIN, pOLEOSIN could be selected. An aleurone-specific promoter may be selected in order to increase growth at the moment of germination, thereby increasing the transport of sugars to the embryo. An inflorescence-specific promoter, such as pLEAFY, may be utilised if the desired outcome would be to modify the number of flower organs. To produce male-sterile plants one would need an anther specific promoter. To impact on flower architecture for example petal size, one could choose a petal-specific promoter. If the desired outcome would be to modify growth and/or developmental characteristics in particular organs, then the choice of the promoter would depend on the organ to be modified. For example, use of a root-specific promoter would lead to increased growth and/or increased biomass or yield of the root and/or phenotypic alteration of the root. This would be particularly important where it is the root itself that is the desired end product, such crops include sugar beet, turnip, carrot, and potato. A fruit-specific promoter may be used to modify, for example, the strength of the outer skin of the fruit or to increase the size of the fruit. A green tissue-specific promoter may be used to increase leaf size. A cell wall-specific promoter may be used to increase the rigidity of the cell wall, thereby increasing

pathogen resistance. An anther-specific promoter may be used to produce male-sterile plants. A vascular-specific promoter may be used to increase transport from leaves to seeds. A nodule-specific promoter may be used to increase the nitrogen fixing capabilities of a plant, thereby increasing the nutrient levels in a plant. A stress-inducible promoter may also be used to drive expression of a nucleic acid to increase membrane integrity during conditions of stress. A stress inducible promoter such as the water stress induced promoter WSI18, the drought stress induced, the ABA related promoter rab21 or any other promoter which is induced under a particular stress condition such as temperature stress (cold, freezing, heat) or osmotic stress, or drought stress or oxidative stress or biotic stress can be used to drive expression of metallothionein. Plants of commercial interest, such as rice or corn, transformed with this construct may show enhanced growth, enhanced yield, increased biomass and increased survival potential under stress conditions and stress tolerance and pathogen resistance when compared to plants not having the plant metallothionein gene under the control of a stress inducible promoter.

Preferably, the nucleic acid sequence capable of modulating expression of a gene encoding a metallothionein is operably linked to a constitutive promoter. The term "constitutive" as defined herein refers to a promoter that is expressed predominantly in at least one tissue or organ and predominantly at any life stage of the plant. Preferably the promoter is expressed predominantly throughout the plant. Preferably, the constitutive promoter is the GOS2 promoter from rice.

Optionally, one or more terminator sequences may also be used in the construct introduced into a plant. The term "terminator" encompasses a control sequence which is a DNA sequence at the end of a transcriptional unit which signals 3' processing and polyadenylation of a primary transcript and termination of transcription. Additional regulatory elements may include transcriptional as well as translational enhancers. Those skilled in the art will be aware of terminator and enhancer sequences which may be suitable for use in performing the invention. Such sequences would be known or may readily be obtained by a person skilled in the art.

The genetic constructs of the invention may further include an origin of replication sequence which is required for maintenance and/or replication in a specific cell type. One example is when a genetic construct is required to be maintained in a bacterial cell as an episomal genetic element (e.g. plasmid or cosmid molecule). Preferred origins of replication include, but are not limited to, the f1-ori and colE1.

The genetic acid construct may optionally comprise a selectable marker gene. As used herein, the term "selectable marker gene" includes any gene which confers a phenotype on a cell in which it is expressed to facilitate the identification and/or selection of cells which are transfected or transformed with a nucleic acid construct of the invention. Suitable markers may be selected from markers that confer antibiotic or herbicide resistance. Cells containing the recombinant DNA will thus be able to survive in the presence of antibiotic or herbicide concentrations that kill untransformed cells. Examples of selectable marker genes include the bar gene which provides resistance to the herbicide Basta; the npt gene which confers resistance to the antibiotic kanamycin; the hpt gene which confers hygromycin resistance. Visual markers, such as the Green Fluorescent Protein (GFP, Haseloff *et al.*, Nature 334, 585 – 591, 1997), β-glucuronidase (GUS) or luciferase may also be used as selectable markers. Further examples of suitable selectable marker genes include the ampicillin resistance (Ampr), tetracycline resistance gene (Tcr), bacterial kanamycin resistance gene (Kanr), phosphinothricin resistance gene, neomycin phosphotransferase gene (nptII), hygromycin resistance gene, gene, and the chloramphenicol acetyltransferase (CAT) gene, amongst others.

The present invention also encompasses plants obtainable by the methods according to the present invention. The present invention therefore provides plants obtainable by the method according to the present invention, which plants have modified growth characteristics and which plants have altered metallothionein activity and/or altered expression of a nucleic acid sequence encoding a metallothionein.

According to a third embodiment of the present invention, there is provided a method for the production of transgenic plants having modified growth characteristics, comprising introduction and expression in a plant of a nucleic acid molecule of the invention.

More specifically, the present invention provides a method for the production of transgenic plants having modified growth characteristics, which method comprises:

(i) introducing into a plant or plant cell a nucleic acid sequence or a portion thereof encoding metallothionein or a homologue, derivative or active fragment thereof;

(ii) cultivating the plant cell under conditions promoting regeneration and mature plant growth.

The protein itself and/or the nucleic acid itself may be introduced directly into a plant cell or into the plant itself (including introduction into a tissue, organ or any other part of the plant). According to a preferred feature of the present invention, the nucleic acid sequence is

preferably introduced into a plant by transformation. The nucleic acid sequence is preferably as represented by SEQ ID NO: 1 or a portion thereof or sequences capable of hybridising therewith, or is a nucleic acid sequence encoding an amino acid sequence represented by SEQ ID NO: 2 or a homologue, derivative or active fragment thereof. Alternatively, the nucleic acid sequence is as represented by SEQ ID NO: 3 or a portion thereof or sequences capable of hybridising with any of the aforementioned sequences. The amino acid sequence may alternatively be a sequence as represented by SEQ ID NO: 4 or by homologues, derivatives or active fragments thereof.

The term "transformation" as referred to herein encompasses the transfer of an exogenous polynucleotide into a host cell, irrespective of the method used for transfer. Plant tissue capable of subsequent clonal propagation, whether by organogenesis or embryogenesis, may be transformed with a genetic construct of the present invention and a whole plant regenerated therefrom. The particular tissue chosen will vary depending on the clonal propagation systems available for, and best suited to, the particular species being transformed. Exemplary tissue targets include leaf disks, pollen, embryos, cotyledons, hypocotyls, megagametophytes, callus tissue, existing meristematic tissue (e.g., apical meristem, axillary buds, and root meristems), and induced meristem tissue (e.g., cotyledon meristem and hypocotyl meristem). The polynucleotide may be transiently or stably introduced into a host cell and may be maintained non-integrated, for example, as a plasmid. Alternatively, it may be integrated into the host genome. The resulting transformed plant cell can then be used to regenerate a transformed plant in a manner known to persons skilled in the art.

Transformation of a plant species is now a fairly routine technique. Advantageously, any of several transformation methods may be used to introduce the gene of interest into a suitable ancestor cell. Transformation methods include the use of liposomes, electroporation, chemicals that increase free DNA uptake, injection of the DNA directly into the plant, particle gun bombardment, transformation using viruses or pollen and microprojection. Methods may be selected from the calcium/polyethylene glycol method for protoplasts (Krens, F.A. *et al.*, 1882, Nature 296, 72-74; Negrutiu I. *et al.*, June 1987, Plant Mol. Biol. 8, 363-373); electroporation of protoplasts (Shillito R.D. *et al.*, 1985 Bio/Technol 3, 1099-1102); microinjection into plant material (Crossway A. *et al.*, 1986, Mol. Gen Genet 202, 179-185); DNA or RNA-coated particle bombardment (Klein T.M. *et al.*, 1987, Nature 327, 70) infection with (non-integrative) viruses and the like. A preferred method according to the present invention is the protocol according to Hiei *et al.* 1994 in the case of rice transformation.

Generally after transformation, plant cells or cell groupings are selected for the presence of one or more markers which are encoded by plant-expressible genes co-transferred with the gene of interest, following which the transformed material is regenerated into a whole plant.

Following DNA transfer and regeneration, putatively transformed plants may be evaluated, for instance using Southern analysis, for the presence of the gene of interest, copy number and/or genomic organisation. Alternatively or additionally, expression levels of the newly introduced DNA may be monitored using Northern and/or Western analysis, both techniques being well known to persons having ordinary skill in the art.

The generated transformed plants may be propagated by a variety of means, such as by clonal propagation or classical breeding techniques. For example, a first generation (or T1) transformed plant may be selfed to give homozygous second generation (or T2) transformants, and the T2 plants further propagated through classical breeding techniques.

The generated transformed organisms may take a variety of forms. For example, they may be chimeras of transformed cells and non-transformed cells; clonal transformants (e.g., all cells transformed to contain the expression cassette); grafts of transformed and untransformed tissues (e.g., in plants, a transformed rootstock grafted to an untransformed scion).

The present invention clearly extends to any plant cell or plant produced by any of the methods described herein, and to all plant parts and propagules thereof. The present invention extends further to encompass the progeny of a primary transformed or transfected cell, tissue, organ or whole plant that has been produced by any of the aforementioned methods, the only requirement being that progeny exhibit the same genotypic and/or phenotypic characteristic(s) as those produced in the parent by the methods according to the invention. The invention also includes host cells containing an isolated nucleic acid molecule encoding a protein capable of modulating a metallothionein, preferably wherein the protein is a metallothionein. Preferred host cells according to the invention are plant cells. The invention also extends to harvestable parts of a plant such as but not limited to seeds, leaves, fruits, flowers, stem cultures, rhizomes, tubers and bulbs.

The term "plant" as used herein encompasses whole plants, ancestors and progeny of the plants and plant parts, including seeds, shoots, stems, roots (including tubers), and plant cells, tissues and organs. The term "plant" also therefore encompasses suspension cultures, embryos, meristematic regions, callus tissue, leaves, seeds, roots, shoots, gametophytes, sporophytes, pollen, and microspores. Plants that are particularly useful in the methods of the

invention include all plants which belong to the superfamily Viridiplantae, in particular monocotyledonous and dicotyledonous plants including a fodder or forage legume, ornamental plant, food crop, tree, or shrub selected from the list comprising *Acacia* spp., *Acer* spp., *Actinidia* spp., *Aesculus* spp., *Agathis australis*, *Albizia amara*, *Alsophila tricolor*, *Andropogon* spp., *Arachis* spp, *Areca catechu*, *Astelia fragrans*, *Astragalus cicer*, *Baikiaea plurijuga*, *Betula* spp., *Brassica* spp., *Bruguiera gymnorrhiza*, *Burkea africana*, *Butea frondosa*, *Cadaba farinosa*, *Calliandra* spp, *Camellia sinensis*, *Canna indica*, *Capsicum* spp., *Cassia* spp., *Centroema pubescens*, *Chaenomeles* spp., *Cinnamomum cassia*, *Coffea arabica*, *Colophospermum mopane*, *Coronillia varia*, *Cotoneaster serotina*, *Crataegus* spp., *Cucumis* spp., *Cupressus* spp., *Cyathea dealbata*, *Cydonia oblonga*, *Cryptomeria japonica*, *Cymbopogon* spp., *Cynthea dealbata*, *Cydonia oblonga*, *Dalbergia monetaria*, *Davallia divaricata*, *Desmodium* spp., *Dicksonia squarosa*, *Diheteropogon amplectens*, *Dioclea* spp, *Dolichos* spp., *Dorycnium rectum*, *Echinochloa pyramidalis*, *Ehrartia* spp., *Eleusine coracana*, *Eragrestis* spp., *Erythrina* spp., *Eucalyptus* spp., *Euclea schimperi*, *Eulalia villosa*, *Fagopyrum* spp., *Feijoa sellowiana*, *Fragaria* spp., *Flemingia* spp, *Freycinetia banksii*, *Geranium thunbergii*, *Ginkgo biloba*, *Glycine javanica*, *Gliricidia* spp, *Gossypium hirsutum*, *Grevillea* spp., *Guibourtia coleosperma*, *Hedysarum* spp., *Hemarthia altissima*, *Heteropogon contortus*, *Hordeum vulgare*, *Hyparrhenia rufa*, *Hypericum erectum*, *Hyperthelia dissoluta*, *Indigo incarnata*, *Iris* spp., *Leptarrhena pyrolifolia*, *Lespediza* spp., *Lettuca* spp., *Leucaena leucocephala*, *Loudetia simplex*, *Lotonus bainesii*, *Lotus* spp., *Macrotyloma axillare*, *Malus* spp., *Manihot esculenta*, *Medicago sativa*, *Metasequoia glyptostroboides*, *Musa sapientum*, *Nicotianum* spp., *Onobrychis* spp., *Ornithopus* spp., *Oryza* spp., *Peltophorum africanum*, *Pennisetum* spp., *Persea gratissima*, *Petunia* spp., *Phaseolus* spp., *Phoenix canariensis*, *Phormium cookianum*, *Photinia* spp., *Picea glauca*, *Pinus* spp., *Pisum sativum*, *Podocarpus totara*, *Pogonarthria fleckii*, *Pogonarthria squarrosa*, *Populus* spp., *Prosopis cineraria*, *Pseudotsuga menziesii*, *Pterolobium stellatum*, *Pyrus communis*, *Quercus* spp., *Rhaphiolepsis umbellata*, *Rhopalostylis sapida*, *Rhus natalensis*, *Ribes grossularia*, *Ribes* spp., *Robinia pseudoacacia*, *Rosa* spp., *Rubus* spp., *Salix* spp., *Schyzachyrium sanguineum*, *Sciadopitys verticillata*, *Sequoia sempervirens*, *Sequoiadendron giganteum*, *Sorghum bicolor*, *Spinacia* spp., *Sporobolus fimbriatus*, *Stiburus alopecuroides*, *Stylosanthos humilis*, *Tadehagi* spp, *Taxodium distichum*, *Themeda triandra*, *Trifollum* spp., *Triticum* spp., *Tsuga heterophylla*, *Vaccinium* spp., *Vicia* spp., *Vitis vinifera*, *Watsonia pyramidata*, *Zantedeschia aethiopica*, *Zea mays*, amaranth, artichoke, asparagus, broccoli, Brussels sprouts, cabbage, canola, carrot, cauliflower, celery, collard greens, flax, kale, lentil, oilseed rape, okra, onion, potato, rice, soybean, straw, sugar beet, sugar cane, sunflower, tomato, squash tea, trees and algae amongst others. According to a preferred feature of the present invention, the plant is a monocotyledonous plant, further preferably a cereal, most preferably a plant selected from

rice, maize, wheat, barley, soybean, sunflower, canola, sugarcane, alfalfa, millet, barley, rapeseed and cotton.

Advantageously, performance of the method according to the present invention results in plants having a variety of modified growth characteristics, such modified growth characteristics including increased yield/biomass with the proviso that increased yield is not an increased iron concentration, modified architecture, modified stress response with the proviso that the stress is not cold stress, osmotic stress, saline stress or stress caused by pathogens, and faster growth, each relative to corresponding wild type plants

The term "increased yield" encompasses an increase in biomass in one or more parts of a plant relative to the biomass of corresponding wild-type plants. The term also encompasses an increase in seed yield, which includes an increase in the biomass of the seed (seed weight) and/or an increase in the number of (filled) seeds and/or in the size of the seeds and/or an increase in seed volume, each relative to corresponding wild-type plants. An increase in seed size and/or volume may also influence the composition of seeds. An increase in seed yield could be due to an increase in the number and/or size of flowers. An increase in yield might also increase the harvest index, which is expressed as a ratio of the total biomass over the yield of harvestable parts, such as seeds. An increase in yield also encompasses a better performance of the plant under non-stress conditions or under stress conditions compared to wild-type plants. Stress conditions include any type of environmental stress and biotic and abiotic stresses.

According to a preferred feature of the present invention, performance of the methods according to the present invention result in plants having modified yield. Preferably, the modified yield includes an increased total number of seeds, increased total weight of seeds and/or an increase of the Thousand Kernel Weight, each relative to control plants. Therefore, according to the present invention, there is provided a method for increasing yield of plants, which method comprises modulating expression of a nucleic acid sequence encoding a metallothionein and/or modulating activity of the metallothionein itself in a plant, preferably wherein the metallothionein is encoded by a nucleic acid sequence represented by SEQ ID NO: 1 or a portion thereof or sequences capable of hybridising therewith or wherein the metallothionein is represented by SEQ ID NO: 2 or a homologue, derivative or active fragment thereof. Alternatively, the metallothionein may be encoded by a nucleic acid sequence represented by SEQ ID NO: 3, or by a portion thereof or by sequences capable of hybridising therewith, or wherein the metallothionein is represented by SEQ ID NO: 4, or a homologue, derivative or active fragment of any thereof.

"Modified architecture" may be due to change in cell division. The term "architecture" as used herein encompasses the appearance or morphology of a plant, including any one or more structural features or combination of structural features thereof. Such structural features include the shape, size, number, position, texture, arrangement, and pattern of any cell, tissue or organ or groups of cells, tissues or organs of a plant, including the root, leaf, shoot, stem or tiller, petiole, trichome, flower, inflorescence (for monocots and dicots), panicles, petal, stigma, style, stamen, pollen, ovule, seed, embryo, endosperm, seed coat, aleurone, fibre, cambium, wood, heartwood, parenchyma, aerenchyma, sieve element, phloem or vascular tissue, amongst others. Modified architecture therefore includes all aspects of modified growth of the plant. Sometimes plants modify their architecture in response to certain conditions such as stress and pathogens (e.g. fungi). Therefore, within the scope of the term "architecture" is included modified architecture under conditions such as stress and pathogens.

Preferably, the modified architecture includes modified number of primary panicles, modified plant height and modified total area, each relative to control plants. Therefore, according to the present invention, there is provided a method for modifying the architecture of plants, particularly the number of primary panicles, plant height and plant area, which method comprises modulating expression of a nucleic acid sequence encoding a metallothionein and/or modulating activity of the metallothionein itself in a plant, preferably wherein the metallothionein is encoded by a nucleic acid sequence represented by SEQ ID NO: 1 or a portion thereof or sequences capable of hybridising therewith or wherein the metallothionein is represented by SEQ ID NO: 2 or a homologue, derivative or active fragment thereof. Alternatively, the metallothionein may be encoded by a nucleic acid sequence represented by SEQ ID NO: 3, or by a portion thereof or by sequences capable of hybridising with the aforementioned sequences, or wherein the metallothionein is represented by SEQ ID NO: 4, or a homologue, derivative or active fragment thereof.

The present invention also relates to use of a nucleic acid sequence encoding a metallothionein and homologues, derivatives and active fragments thereof in modifying the growth characteristics of plants, preferably in increasing yield and modifying plant architecture, with the proviso that increased yield is not an increased iron concentration. The nucleic acid sequence is preferably as represented by SEQ ID NO: 1 or a portion thereof or sequences capable of hybridising therewith or is an amino acid sequence represented by SEQ ID NO: 2 or a homologue, derivative or active fragment thereof.

The present invention also relates to use of a nucleic acid sequence encoding a metallothionein and homologues, derivatives and active fragments thereof and to use of the metallothionein itself and to homologues, derivatives and active fragments thereof as a growth regulator. The nucleic acid sequences hereinbefore described (and portions of the same and sequences capable of hybridising with the same) and the amino acid sequences hereinbefore described (and homologues, derivatives and active fragments of the same) are useful in modifying the growth characteristics of plants, as hereinbefore described. The sequences would therefore find use as growth regulators, such as herbicides or growth stimulators. The present invention also provides a composition comprising a protein represented by any of the aforementioned amino acid sequences or homologues, derivatives or active fragments thereof for the use as a growth regulator.

Conversely, the sequences according to the present invention may also be interesting targets for agrochemical compounds, such as herbicides or growth stimulators. Accordingly, the present invention encompasses use of the aforementioned nucleic acid sequences (or a portion of the same or sequences capable of hybridising with the same) or an amino acid sequence as hereinbefore described (or homologues, derivatives and active fragments of the same) as targets for an agrochemical compound, such as a herbicide or a growth stimulator.

The methods according to the present invention may also be practised by co-expression of a gene encoding a metallothionein protein in a plant with at least one other gene that cooperates with the gene encoding a metallothionein protein. Such a gene may be any other metallothionein or other metal binding protein or other protein having an essential role in modulating metal concentrations in a plant. Co-expression may be effected by cloning the genes under the control of a plant expressible promoter in a plant expressible vector and introducing the expression vector(s) into a plant cell using *Agrobacterium*-mediated plant transformation.

The methods according to the present invention result in plants having modified growth characteristics, as described hereinbefore. These advantageous growth characteristics may also be combined with other economically advantageous traits, such as further yield-enhancing traits, tolerance to various stresses, traits modifying various architectural features and/or biochemical and/or physiological features.

Description of figures

The present invention will now be described with reference to the following figures in which:

089-MT-PROV

Figure 1: Schematic presentation of the entry clone p3372, containing CDS1585 within the AttL1 and AttL2 sites for Gateway® cloning in the pDONR201 backbone. CDS1585 is the internal code for the *Arabidopsis thaliana* metallothionein-like AtMT2a coding sequence. This vector contains also a bacterial kanamycine-resistance cassette and a bacterial origin of replication.

Figure 2: Binary vector for the expression in *Oryza sativa* of the *Arabidopsis thaliana* metallothionein-like *AtMT2a* gene (CDS1585) under the control of the rice GOS2 promoter (PRO0129). This vector contains a T-DNA derived from the Ti Plasmid, limited by a left border (LB repeat, LB Ti C58) and a right border (RB repeat, RB Ti C58)). From the left border to the right border, this T-DNA contains: a CaMV35S promoter – *hpt* CDS – CaMV35S terminator cassette for antibiotic selection of transformed plants; a CaMV35S promoter – *GFP* CDS – NOS terminator cassette for visual screening of transformed plants; the PRO0129 - CDS1585 - *zein* and *rbcS-deltaGA* double terminator cassette for expression of the *Arabidopsis thaliana* metallothionein-like *AtMT2a* gene. This vector also contains an origin of replication from pBR322 for bacterial replication and a selectable marker (Spe/SmeR) for bacterial selection with spectinomycin and streptomycin.

Figure 3: Sequence listing

Examples

The present invention will now be described with reference to the following examples, which are by way of illustration alone.

DNA manipulation: unless otherwise stated, recombinant DNA techniques are performed according to standard protocols described in (Sambrook (2001) Molecular Cloning: a laboratory manual, 3rd Edition Cold Spring Harbor Laboratory Press, CSH, New York) or in Volumes 1 and 2 of Ausubel *et al.* (1984), Current Protocols in Molecular Biology, Current Protocols. Standard materials and methods for plant molecular work are described in Plant Molecular Biology Labfase (1993) by R.D.D. Croy, published by BIOS Scientific Publications Ltd (UK) and Blackwell Scientific Publications (UK).

Example 1. Cloning of CDS0851

The *Arabidopsis* metallothionein AtMT2a (CDS1585) was amplified by PCR using as template an *Arabidopsis thaliana* seedling cDNA library (Invitrogen, Paisley, UK). After reverse transcription of RNA extracted from seedlings, the cDNAs were cloned into pCMV Sport 6.0. Average insert size of the bank was 1.5 kb, and original number of clones was of 1.59×10^7 cfu.

The original titer was determined to be $9.6x10^5$ cfu/ml, and after a first amplification became $6x10^{11}$ cfu/ml. After plasmid extraction, 200 ng of template was used in a 50 µl PCR mix. Primers prm03240 (SEQ ID NO 5) and prm03241 (SEQ ID NO 6), which include the AttB sites for Gateway recombination, were used for PCR amplification. PCR was performed using Hifi Taq DNA polymerase in standard conditions. A PCR fragment of 246 bp was amplified and purified also using standard methods. The first step of the Gateway procedure, the BP reaction, was then performed, during which the PCR fragment recombines in vivo with the pDONR201 plasmid to produce, according to the Gateway terminology, an "entry clone", p3372 (Figure 1). Plasmid pDONR201 was purchased from Invitrogen, as part of the Gateway® technology.

Example 2. Vector construction for transformation with PRO0129-CDS1585 cassette

The entry clone p3372 was subsequently used in an LR reaction with p0640, a destination vector used for *Oryza sativa* transformation. This vector contains as functional elements within the T-DNA borders: a plant selectable marker; a GFP expression cassette; and a Gateway cassette intended for LR *in vivo* recombination with the sequence of interest already cloned in the entry clone. A rice GOS2 promoter for constitutive expression (PRO0129) is located upstream of this Gateway cassette.

After the LR recombination step, the resulting expression vector p3436 (Figure 2) can be transformed into the *Agrobacterium* strain LBA4044 and subsequently to *Oryza sativa* plants.

Example 3. Transformation of rice with PRO0129 - CDS1585

Mature dry seeds of *Oryza sativa* japonica cultivar Nipponbare were dehusked. Sterilization was done by incubating the seeds for one minute in 70% ethanol, followed by 30 minutes in 0.2% $HgCl_2$ and by 6 washes of 15 minutes with sterile distilled water. The sterile seeds were then germinated on a medium containing 2,4-D (callus induction medium). After a 4-week incubation in the dark, embryogenic, scutellum-derived calli were excised and propagated on the same medium. Two weeks later, the calli were multiplied or propagated by subculture on the same medium for another 2 weeks. 3 days before co-cultivation, embryogenic callus pieces were sub-cultured on fresh medium to boost cell division activity. The *Agrobacterium* strain LBA4044 harbouring the binary vector p3076 was used for co-cultivation. The *Agrobacterium* strain was cultured for 3 days at 28°C on AB medium with the appropriate antibiotics. The bacteria were then collected and suspended in liquid co-cultivation medium at an OD_{600} of about 1. The suspension was transferred to a petri dish and the calli were immersed in the suspension during 15 minutes. Next, the callus tissues were blotted dry on a

filter paper, transferred to solidified co-cultivation medium and incubated for 3 days in the dark at 25°C.

Hereafter, co-cultivated callus was grown on 2,4-D-containing medium for 4 weeks in the dark at 28°C in the presence of a selective agent at a suitable concentration. During this period, rapidly growing resistant callus islands developed. Upon transfer of this material to a regeneration medium and incubation in the light, the embryogenic potential was released and shoots developed in the next four to five weeks. Shoots were excised from the callus and incubated for 2 to 3 weeks on an auxin-containing medium from which they were transferred to soil. Hardened shoots were grown under high humidity and short days in a greenhouse. Finally seeds were harvested three to five months after transplanting. The method yielded single locus transformants at a rate of over 50 % (Aldemita and Hodges, 1996, Chan *et al.*, 1993, Hiei *et al.*, 1994).

Example 4. Evaluation of transgenic rice transformed with PRO0129-CDS1585

Approximately 15 to 20 independent T0 rice transformants were generated. The primary transformants were transferred from tissue culture chambers to a greenhouse for growing and harvest of T1 seed. 7 events, of which the T1 progeny segregated 3:1 for presence/absence of the transgene, were retained. For each of these events, approximately 10 T1 seedlings containing the transgene (hetero- and homo-zygotes), and approximately 10 T1 seedlings lacking the transgene (nullizygotes), were selected by monitoring GFP expression.

Vegetative growth measurements:

The selected T1 plants (approximately 10 with the transgene and approximately 10 without the transgene) were transferred to a greenhouse. Each plant received a unique barcode label to link unambiguously the phenotyping data to the corresponding plant. The selected T1 plants were grown on soil in 10 cm diameter pots under the following environmental settings: photoperiod= 11.5 h, daylight intensity= 30,000 lux or more, daytime temperature= 28°C or higher, night time temperature= 22°C, relative humidity= 60-70%. Transgenic plants and the corresponding nullizygotes were grown side-by-side at random positions. From the stage of sowing until the stage of maturity each plant were passed several times through a digital imaging cabinet and imaged. At each time point digital images (2048x1536 pixels, 16 million colours) were taken of each plant from at least 6 different angles. The parameters described below were derived in an automated way from the all the digital images of all the plants, using image analysis software.

(i) Above ground plant area: plant above ground area was determined by counting the total number of pixels from aboveground plant parts discriminated from the background. This value

was averaged for the pictures taken on the same time point from the different angles and was converted to a physical surface value expressed in square mm by calibration. Experiments show that the aboveground plant area measured this way correlates with the biomass of plant parts above ground.

(ii) Plant height: height was determined by the distance between the horizontal lines going through the upper pot edge and the uppermost pixel corresponding to a plant part above ground. This value was averaged for the pictures taken on the same time point from the different angles and was converted, by calibration, to a physical distance expressed in mm. Experiments showed that plant height measured this way correlate with plant height measured manually with a ruler.

(iii) Number of primary panicles: the tallest panicle and all the panicles that overlapped with the tallest panicle when aligned vertically were considered as primary panicles, and counted manually.

Seed-related parameter measurements:
The mature primary panicles were harvested, bagged, barcode-labeled and then dried for three days in the oven at 37°C. The panicles were then threshed and all the seeds were collected and counted. The filled husks were separated from the empty ones using an air-blowing device. The empty husks were discarded and the remaining fraction was counted again. The filled husks were weighed on an analytical balance. This procedure resulted in the set of seed-related parameters described below.

(i) Total seed number per plant: was measured by counting the number of husks harvested from a plant.

(ii) Total seed yield per plant: the yield was measured by weighing all filled husks harvested from a plant.

(iii) Thousand Kernel Weight (TKW): this parameter is extrapolated from the number of filled seeds counted, and their total weight.

Statistical analysis: t-test and F-test
A two factor ANOVA (analysis of variants) was used as statistical model for the overall evaluation of plant phenotypic characteristics. An F-test was carried out on all the parameters measured of all the plants of all the events transformed with the gene of the present invention.

The F-test is carried out to check for an effect of the gene over all the transformation events and to verify for an overall effect of the gene, also named herein "global gene effect". If the value of the F-test shows that the data are significant, than it is concluded that there is a "gene" effect, meaning that not only presence or the position of the gene is causing the differences in phenotype. The threshold for significance for a true global gene effect is set at 5% probability level for the F-test.

To check for an effect of the genes within an event, i.e., for a line-specific effect, a t-test was performed within each event using data sets from the transgenic plants and the corresponding null plants. "Null plants" or "Null segregants" are the plants treated in the same way as the transgenic plant, but from which the transgene has segregated. Null plants can also be described as the homozygous negative transformants. The threshold for significance for the t-test is set at 10% probability level. Within one population of 5 transformation events, some events can be under or above this t-test threshold. This is based on the hypothesis that a gene might only have an effect in certain positions in the genome, and that the occurrence of this position-dependent effect is not uncommon. This kind of gene effect is also named herein a "line effect of the gene".
The p-value is obtained by comparing the t-value to the t-distribution or alternatively, by comparing the F-value to the F-distribution. The p-value then stands for the probability that the null hypothesis (null hypothesis being "there is no effect of the transgene") is correct.

Example 5. Results of the evaluation of transgenic plants transformed with PRO0129-CDS1585

I. Vegetative parameters

Total area:

Results are presented in table 1. The presence of the transgene has a very significant overall positive effect on aboveground area of the transgenics, when compared to their null segregants. The transgenic plants have an increase in total area of 17 % compared to the null segregants, with a probability of both populations being equal of only 0.0048. In addition to this overall positive effect, two specific transformation events, 84374 and 84384, also present significant aboveground area increase corresponding to 31 and 55%, with p-values for the t-test of 0.0652 and 0.0045 respectively.

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Table 1:

Total Area					
Line	TR	null	dif	% dif	p-value
84162	55657	48162	7495	16	0.2654
84357	50639	46473	4166	9	0.5157
84374	54361	41388	12973	31	0.0652
84384	53809	34788	19021	55	0.0045
84417	55286	50325	4961	10	0.4391
95271	35977	35183	794	2	0.9033
95273	38819	37240	1579	4	0.8141
Overall	**49107**	**42109**	**6998**	**17**	**0.0048**

Each row corresponds to one event, for which average maximum aboveground area (expressed in mm^2) was determined for the 10 transgenics (TR) and the 10 null lines (null). The numeric difference between the positive plants and the negative plants is given (dif) as well as the percentage of difference between these plants (% dif), p-values stand for the probability produced by the t-test for each plant line. The last row presents the average numbers for all seven events. There, the p-value stands for the p-value derived from the F-test.

Primary panicles:

Results are presented in table 2. The two events positive for aboveground area are also positive for the number of first panicles that developed. The p-value of the t-test is respectively 0.0009 and 0.0297 for events 84374 and 84384. The p-value obtained from the F-test to check for overall gene effect is not strongly significant (0.0958), although a 16% increase can be observed when the transgene is present.

Table 2:

First pan					
Line	TR	null	dif	% dif	p-value
84162	3.8	3.3	0.5	15	0.4817
84357	3.6	4.4	-0.8	-18	0.2233
84374	5.9	3.5	2.4	69	0.0009
84384	4.9	3.4	1.5	45	0.0297
84417	4	3.4	0.6	18	0.3604
95271	3.6	2.8	0.8	30	0.2139
95273	3.2	4	-0.8	-19	0.2637
Overall	**4.1**	**3.6**	**0.6**	**16**	**0.0958**

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Each row corresponds to one event, for which the average number of first panicles has been determined for the 10 transgenics (TR) and the 10 null lines (null), expressed in units. The numeric difference between the positive plants and the negative plants is given (dif) as well as the percentage of difference between these plants (% dif). P-values stand for the probability produced by the t-test for each plant line. The last row presents the average numbers for all seven events. There, the p-value stands for the p-value derived from the F-test.

Height:

Results are presented in table 3. Again, the same two events positive for aboveground area and number of first panicles, are positive for height, with p-values for the t-test of 0.0256 and 0.0215, respectively. Another event, 84417, is also positive for this parameter, with a p-value of 0.0984. More importantly, a very significant overall effect of the transgene on plant height can be observed, with a p-value from the F-test being 0.0034.

Table 3:

Height					
Line	TR	null	dif	% dif	p-value
84162	1069	1036	33	3	0.4994
84357	991	932	59	6	0.2063
84374	1012	898	114	13	0.0256
84384	954	843	111	13	0.0215
84417	1074	996	77	8	0.0984
95271	895	882	13	2	0.7786
95273	911	938	-27	-3	0.5762
Overall	**985**	**933**	**53**	6	**0.0034**

Each row corresponds to one event, for which the average total number of seeds has been determined for the 10 transgenics (TR) and the 10 null lines (null), expressed in mm. The numeric difference between the positive plants and the negative plants is given (dif) as well as the percentage of difference between these plants (% dif). P-values stand for the probability produced by the t-test for each plant line. The last row presents the average numbers for all seven events. There, the p-value stands for the p-value derived from the F-test.

II. Seed-related parameters

Total number of seeds:

Results are presented in table 4. The two events positive for several vegetative parameters, are also positive for the total number of seeds that were produced: event 84374 showed an increase of 55% over the nullizygotes and event 84384 produced 43% more seeds. These increases were significantly, as the t-test produced p-values of 0.0085 and 0.0541

respectively. More importantly, the presence of the transgene has a significant positive overall effect with a p-value of 0.0404, indicating that the transgenic plants produce more seeds than their null counterparts.

Table 4:

nrtotalseed					
Line	TR	null	dif	% dif	p-value
84162	371.5	323.9	47.6	15	0.3955
84357	307	338.6	-31.6	-9	0.554
84374	437	281.7	155.33	55	0.0085
84384	364.7	254.9	109.83	43	0.0541
84417	358.2	337.4	20.8	6	0.6968
95271	250.7	210.4	40.29	19	0.46
95273	252.2	271.7	-19.56	-7	0.7267
Overall	**335.1**	**292.8**	**42.3**	**14**	**0.0404**

Each row corresponds to one event, for which the average total number of seeds has been determined for the 10 transgenics (TR) and the 10 null lines (null), expressed in units. The numeric difference between the positive plants and the negative plants is given (dif) as well as the percentage of difference between these plants (% dif). P-values stand for the probability produced by the t-test for each plant line. The last row presents the average numbers for all seven events. There, the p-value stands for the p-value derived from the F-test.

Total weight of seeds:

Results are presented in table 5. Event 84384 shows furthermore a higher seed yield (56% increase), which is a significant difference as the p-value from the t-test is 0.0528. The overall effect of the presence of the transgene on seed yield shows a 9% increase.

Table 5:

Total wg seeds					
Line	TR	null	dif	% dif	p-value
84162	4.9	4.3	0.55	13	0.6471
84357	3.7	3.6	0.09	3	0.6819
84374	3	2.7	0.29	11	0.8955
84384	3.1	2	1.12	56	0.0528
84417	3.8	4.4	-0.59	-13	0.8517
95271	2.1	1.4	0.67	49	0.4168
95273	2.2	2.4	-0.14	-6	0.8443
Overall	**3.3**	**3**	**0.26**	**9**	**0.2092**

Each row corresponds to one event, for which the average total seed weight has been determined for the 10 transgenics (TR) and the 10 null lines (null), expressed in grams. The numeric difference between the positive plants and the negative plants is given (dif) as well as the percentage of difference between these plants (% dif). P-values stand for the probability produced by the t-test for each plant line. The last row presents the average numbers for all seven events. There, the p-value stands for the p-value derived from the F-test.

Thousand Kernel Weight:

Results are presented in table 6. One of the events previously identified as positive for a number of parameters (84374), is also positive for Thousand Kernel Weight (TKW) with an 18% increase in TKW, and a p-value from the T test of 0.0008. More importantly, also the overall effect of the presence of the transgene on TKW is significant, with a p-value from the F-test of 0.0149, showing that seeds from the transgenics are heavier.

Table 6:

TKW					
Line	TR	null	dif	% dif	p-value
84162	25.3	25.6	-0.27	-1	0.7752
84357	25.3	24.8	0.51	2	0.5871
84374	23.8	20.1	3.64	18	0.0008
84384	24.7	24.1	0.64	3	0.5561
84417	24.4	23.2	1.29	6	0.1557
95271	23.2	22.4	0.8	4	0.387
95273	23.1	22.9	0.13	1	0.8877
Overall	**24.3**	**23.4**	**0.88**	**4**	**0.0149**

Each row corresponds to one event, for which the average TKW has been determined for the 10 transgenics (TR) and the 10 null lines (null), expressed in units. The numeric difference between the positive plants and the negative plants is given (dif) as well as the percentage of difference between these plants (% dif). P-values stand for the probability produced by the t-test for each plant line. The last row presents the average numbers for all seven events. There, the p-value stands for the p-value derived from the F-test.

From these evaluation data it is clear that there was a variation between the different transformation events (different plant events each transformed with the AtMT2a gene). It is well known to persons skilled in the art, for example a plant molecular biologist, that the expression of transgenes in plants, and hence also the phenotypical effect due to expression of such transgene, can differ dramatically among different independently obtained transgenic lines and progeny thereof. The transgenes present in different independently obtained

transgenic plants differ from each other by the chromosomal insertion locus as well as by the number of transgene copies inserted in that locus and the configuration of those transgene copies in that locus. Differences in expression levels can be ascribed to influence from the chromosomal context of the transgene (the so-called position effect) or from silencing mechanisms triggered by certain transgene configurations (e.g. inwards facing tandem insertions of transgenes are prone to silencing at the transcriptional or post-transcriptional level).

The exact configuration and insertion loci of the different events have not yet been determined, and expression levels have not been measured. But differences in these will clearly have an impact on the phenotypic parameters that have been measured. In some cases, negative effects may be observed for example when an essential gene is totally silenced instead of being overexpressed (or misexpressed).

Claims

1. Method for modifying plant growth characteristics, comprising modulating expression in a plant of a nucleic acid sequence encoding a metallothionein and/or modulating activity in a plant of a metallothionein.

2. Method according to claim 1, wherein said modulation is effected by recombinant means and/or chemical means.

3. Method according to claim 1 or 2, wherein said modulating expression comprises introducing into a plant a nucleic acid sequence encoding a metallothionein or a homologue, derivative or active fragment thereof.

4. Method according to claim 3, wherein said nucleic acid is a homologous nucleic acid sequence, preferably from a dicotyledonous plant, further preferably from the family Brassicaceae, more preferably the nucleic acid sequence is from *Arabidopsis thaliana*, most preferably as represented by SEQ ID NO: 1 or a portion thereof or sequences capable of hybridising therewith, or a nucleic acid sequence encoding an amino acid sequence represented by SEQ ID NO: 2 or a homologue, derivative or active fragment thereof.

5. Method according to any of claims 1 to 4, wherein said nucleic acid sequence is an alternative splice variant of a nucleic acid sequence encoding a metallothionein or wherein said metallothionein is encoded by a splice variant.

6. Method according to any of claims 1 to 4, wherein said nucleic acid sequence is an allelic variant of a nucleic acid sequence encoding a metallothionein or wherein said metallothionein is encoded by an allelic variant.

7. Method according to any of claims 1 to 4, wherein said nucleic acid sequence is comprised in at least a part of an artificial chromosome, which artificial chromosome preferably also comprises one or more related gene family members.

8. Method according to any of claims 1 to 7, wherein said nucleic acid sequence encoding a metallothionein is overexpressed in a plant.

9. Method according to any of claims 1 to 7, wherein said nucleic acid sequence encoding a metallothionein is downregulated in a plant.

089-MT-PROV

10. Method according to any of claims 1 to 9, wherein expression of said nucleic acid encoding a metallothionein is driven by a constitutive promoter.

11. Method according to any of claims 1 to 10, wherein said modified growth characteristic is selected from any one or more of increased yield/biomass, modified plant architecture, faster growth and modified stress response, each relative to corresponding wild type plants, with the proviso that said increased yield is not an increased iron concentration and that said stress is not cold stress, osmotic stress, saline stress or stress caused by pathogens.

12. Plants obtainable by a method according to any of claims 1 to 11.

13. Construct comprising:

(i) a nucleic acid sequence capable of modulating expression of a nucleic acid encoding a metallothionein and/or activity of a metallothionein;

(ii) one or more control sequence capable of driving expression of the nucleic acid sequence of (i); and optionally

(iii) a transcription termination sequence.

14. Construct according to claim 13, wherein said nucleic acid sequence capable of modulating expression of a nucleic acid encoding a metallothionein and/or activity of a metallothionein is a nucleic acid sequence encoding a metallothionein, preferably as represented by SEQ ID NO: 1 or a portion thereof or by sequences capable of hybridising therewith.

15. Construct according to claim 13 or 14, wherein said control sequences comprise at least a constitutive promoter, preferably a GOS2 promoter.

16. Method for the production of a transgenic plant having modified growth characteristics, which method comprises:

(I) introducing into a plant or plant cell a nucleic acid sequence or a portion thereof encoding a metallothionein or a homologue, derivative or active fragment thereof;

(II) cultivating the plant cell under conditions promoting regeneration and mature plant growth.

17. Transgenic plant having modified growth characteristics, characterised in that said plant has modulated expression in a plant of a nucleic acid sequence encoding a metallothionein and/or modulated activity in a plant of a metallothionein.

18. Transgenic plant according to claim 17, wherein said plant is a monocotyledonous plant, further preferably a cereal, most preferably a plant selected from rice, maize, wheat, barley, soybean, sunflower, canola, sugarcane, alfalfa, millet, barley, rapeseed and cotton.

19. Use of a nucleic acid sequence encoding a metallothionein and homologues, derivatives and active fragments thereof in modifying the growth characteristics of plants, preferably in increasing yield, further preferably seed yield.

20. Use of a metallothionein and homologues, derivatives and active fragments thereof in modifying the growth characteristics of plants.

21. A composition comprising a protein represented by SEQ ID NO 2 or a homologue, derivative or active fragment thereof for the use as a growth regulator.

22. Use of a nucleic acid sequence as represented by SEQ ID NO: 1 or a portion thereof or a sequence represented by SEQ ID NO: 2 or homologues, derivatives and active fragments thereof as targets for an agrochemical compound, such as a herbicide or a growth stimulator.

089-MT-PROV

Abstract

Plants having modified growth characteristics and method for making the same

The present invention concerns a method for modifying the growth characteristics of plants by modulating expression in a plant of a nucleic acid sequence encoding a metallothionein and/or modulating activity in a plant of a metallothionein. The invention also relates to transgenic plants having modified growth characteristics, which plants have modulated expression of a nucleic acid encoding a metallothionein.

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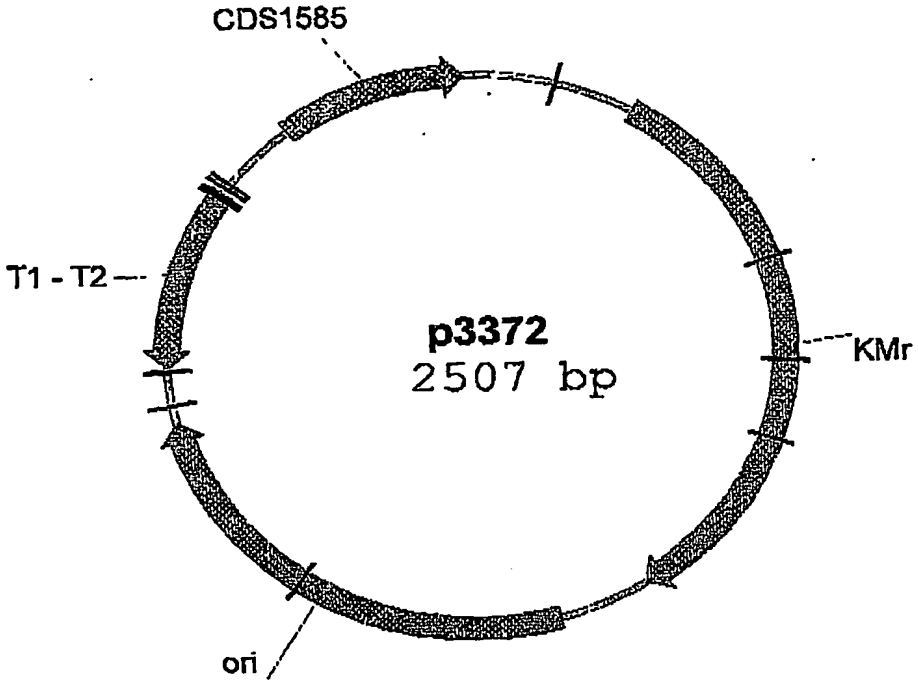

FIGURE 1

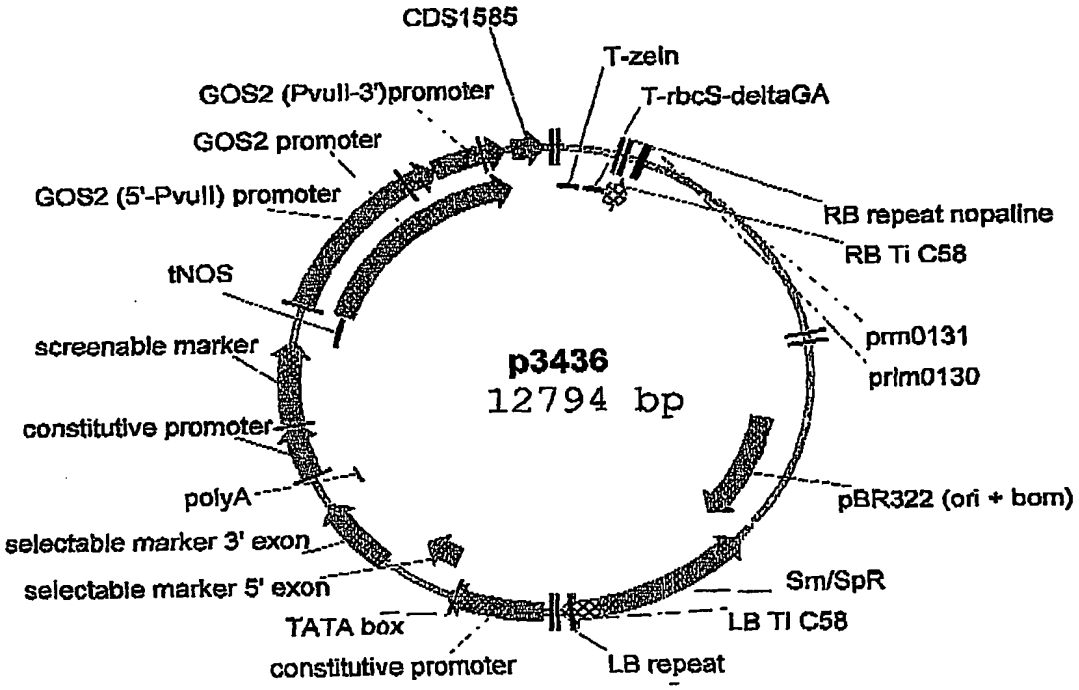

FIGURE 2

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SEQ ID NO 1: AtMT2a (CDS1585) coding sequence (start and stop in bold)

ttttcattcataaatttttcttcaatttgaattttctcgagaaaaa**atg**tcttgctgtggagg
aaactgcggatgtggatctggctgcaagtgcggcaacggttgtggaggttgcaaaatgtacc
ctgacttgggattctccggcgagacaaccacaactgagacttttgtcttgggcgttgcaccg
gcgatgaagaatcagtacgaggcttcaggggagagtaacaacgctgagaacgatgcttgcaa
gtgtggatctgactgcaagtgtgatccttgcacctgcaag**tga**agaagccttttaaataag
cagagataatcgagtctctttaatta

SEQ ID NO 2: AtMT2a (CDS1585) deduced protein sequence

MSCCGGNCGCGSGCKCGNGCGGCKMYPDLGFSGETTTTETFVLGVAPAMKNQYEASGESNNA
ENDACKCGSDCKCDPCTCK

SEQ ID NO 3: *A. thaliana* AtMT-1 cDNA, start and stop codon in bold.

cattcataaatttttcttcaatttgaattttctcgagaaaaa**atg**tcttgctgtggaggaaac
tgcggatgtggatctggctgcaagtgcggcaacggttgtggaggttgcaaaatgtaccctga
cttgggattctccggcgagacaaccacaactgagacttttgtcttgggcgttgcaccggcga
tgaagaatcagtacgaggcttcaggggagagtaacaacgctgagagcgatgcttgcaagtgt
ggatctgactgcaagtgtgatccttgcacctgcaag**tga**agaagccttttaaataagcaga
gataatcgagtctctttaatntaattaagttattcaataagtaaaccatatataggatggtg
tttttaggtttggtttatgtgtaataatggcttcagcttatcttttagccgatcattgtctt
ttgtgtttgttttgatcatatcttttagatgtctagcaaatctgccatgtgatgagtttgta
cttccagtggaatgataatatattatagttttaaatcaaaaaaaaaaa

SEQ ID NO 4: *A. thaliana* AtMT-1 deduced protein sequence

MSCCGGNCGCGSGCKCGNGCGGCKMYPDLGFSGETTTTETFVLGVAPAMKNQYEASGESNNA
ESDACKCGSDCKCDPCTCK

SEQ ID NO 5: prm03240

GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACAATGTCTTGCTGTGGAGGAA

SEQ ID NO 6: prm03241

GGGGACCACTTTGTACAAGAAAGCTGGGTTTCACTTGCAGGTGCAAG

FIGURE 3

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